A Study on Immunological Therapy for Cancer by Using Nitrogen Mustard-treated Cancer Tissue

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Received for Publication Oct. 5, 1965

INTRODUCTION

Although host resistance to cancer is not thoroughly clarified, control of cancer cells by means of immunological procedures has been under investigation for a long time. Many studies have been done in the field of active immunity in cancer[3)7)10)11)14)15)16)17)19)20)]. Judging from recent experimental studies, however, it is generally believed that a spontaneous tumor has not immunizing potency to the autologous host[8)19)24)], although chemically induced tumor e. g. methylcholanthrene-induced sarcoma has specific antigenicity to the host in which the tumor originated[8)12)17)18)19)24)28)]. Attractive attempts have been undertaken to modify or to convert the antigenic structure of cancer cells in order to give them antigenicity to the host[10)14)15)20)26)]. It was previously proved[1)] that nitrogen mustard (HN2), one of the alkylating anticancer agents, endows a new specific antigenicity to proteins to which HN2 is apt to combine. The results led us to propose a working hypothesis that HN2-treated cancer tissue let probably antibody forming cells recognize an altered antigenic substance in the cancer tissue as "not self" and produce antibodies to the modified antigen. The resultant antibodies might concentrate upon the cancer tissue in the presence of specific hapten i. e. nitrogen mustard (HN2). The present study was undertaken to investigate the possibility of this kind of approach in cancer treatment using a spontaneous mammary tumor in an isologous system of mice.

MATERIALS AND METHODS

1) Animals: Female mice of C3H/He line supplied by Kyoto University Inbred Animal Center were employed throughout the study. The mice were one to two months old and were fed ad libitum on a diet of Oriental Solid Chow and water.

2) Tumor and preparation of tumor cell suspension: A mammary adenocarcinoma, CaⅧ, which arose spontaneously in a female of the breeding nucleous of the strain C3H/He was excised, minced and washed in Phosphate Buffered Saline (PBS)** which contained 100 units of penicillin and 100 γ of streptomycin per ml. The minced tissue was then pushed through a wire screen (#60) into a petri dish. The screened tissue was centrifuged at about 700～800 r. p. m. for five minutes and the sediment was

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** Phosphate Buffered Saline (PBS) was prepared according to the following formula: NaCl 16 g, KCl 0.4 g, Na_2HPO_4 2.3 g, KH_2PO_4 0.4 g, distilled H_2O to 800 ml; pH was 7.4.

prepared in a 20% tumor cell suspension by volume. When used for transplantation, 0.2 ml of the suspension was injected subcutaneously into the back of mice of the same inbred strain.

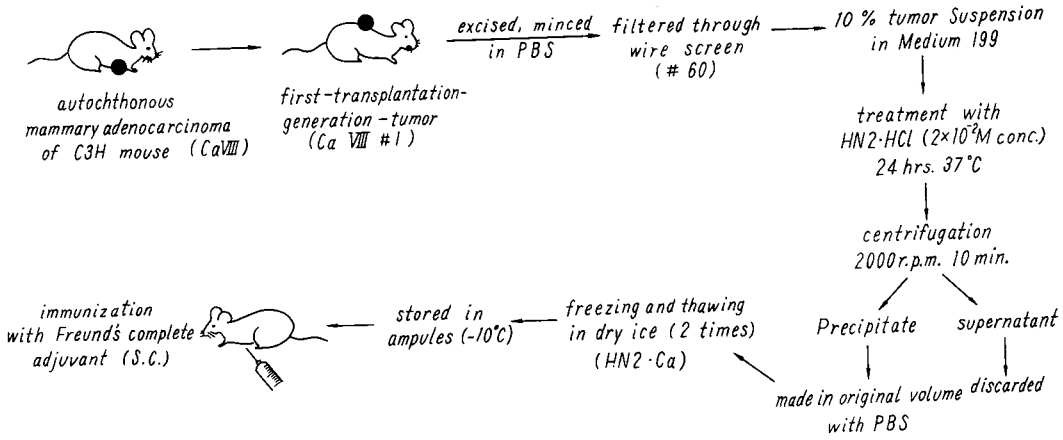

Fig. 1 Preparation of HN2-treated tumor cell suspension

3) In vitro treatment of tumor cell suspension with HN2 (Fig. 1) : A first-transplant-generation tumor of CaVIII (CaVIII ♯1) which was 18×12×10 mm×mm×mm in size, milky white and without hematom or central necrosis, was excised. The excised tissue was screened by the same method as mentioned above and diluted with a synthesized medium, Medium 199, to form a 10% tumor cell suspension. Fifteen mg of nitrogen mustard hydrochloride (HN2•HCl) was dissolved in 1 ml of distilled water and 1/2 N NaOH was added to the HN2 solution to make it free of the base. The solution was mixed with 4 ml of the 10% cell suspension and the mixture was incubated at 37°C for 24 hours. During the incubation, pH of the mixture was kept at 7.2～7.4 by adding 1.4% bicarbonate solution. As a control of the treated cell suspension, another 4 ml of the 10% cell suspension was mixed with 1ml of distilled water and incubated at 37°C for 24 hours. After the incubation, these suspensions were centrifuged at 2000 r. p. m. for 10 minutes. The supernatants were discarded and the precipitates were diluted with PBS to form 10% suspensions. The suspensions were then frozen by using dry ice for 30 minutes and thawed at room temperature. Freezing and thawing were repeated twice. When examined by SCHRECK's method[22], viable cells were hardly found in these suspensions. These preparations were enclosed, in ampules containing 1 ml each and stored at −10°C. The preparations were designated as **"HN2•Ca"** or **"Ca"**, respectively.

4) Immunization : Thirty six animals were divided into three groups,each consisting of 12 mice. The immunization procedure is schematically illustrated in Fig. 2. HN2•Ca or Ca was mixed with an equal volume of FREUND's complete adjuvant which consisted of 8.5 vol. of liquid paraffin, 1.5 vol. of Arlacel A and 4 mg of B. C. G. per ml of liquid paraffin. Each 0.1 ml of the mixtures was inoculated subcutaneously into the flank of two groups of animals twice at an interval of 12 days. Thirty five days after the second inoculation, as a booster injection before challenge, 0.1 ml of HN2•Ca or Ca, without the adjuvant, was given

subcutaneously to the respective groups of mice. As a control, PBS mixed with the adjuvant was inoculated into the third group of mice in the same manner mentioned above.

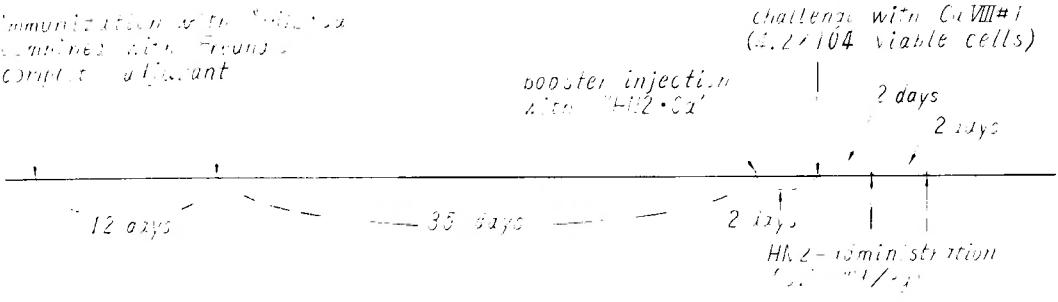

Fig. 2 Immunization, challenge and HN2-administration

5) Challenge : Challenge implant was done 2 days after the last immunization to all of the mice. A 5% Ca VIII # 1 tumor suspension was prepared in the same way as described above. The suspension was laid at 4 C for 15 minutes to sediment large cell clumps. The supernatant was pipetted and its approximate content of viable cells was estimated by SCHRECK's method. Zero point one ml of the suspension containing 4.2×10^4 viable cells, as a challenge implant, was injected subctaneously into the interscapular area of the mice.

6) Administration of HN2 : HN2•HCl was dissolved in distilled water and used within 30 minutes after being prepared. Two days after the challenge implantation, the HN2 solution was administered intraperitoneally every other day, two times, to each half of the three groups which were immunized with HN2•Ca, Ca and PBS, respectively, and these HN2-administered groups were designated as "**HN2•Ca HN2 group**", "**Ca HN2 group**" and "**PBS-HN2 group**", respectively. The dose of HN2 administered was 0.1 mg per Kg body weight, which is about Minimum Effective Dose of HN2 for a mouse[25]. As a control for these HN2-administered groups, the rest of the mice of the respective groups were injected intraperitoneally with 0.2 ml of distilled water instead of the HN2 solution and was designated as "**HN2•Ca group**", "**Ca group**" and "**PBS group**", respectively. The appearance of palpable tumors and their growth were checked from two weeks after challenge once or twice a week for 180 days. The size of a tumor was described as an average of its three main diameters. Mean values of the tumor size of each group were also calculated and were shown as mean growth curve of the group.

RESULTS

Figs. 3, 4 & 5 show the growth curves of the tumors in the groups of mice which were immunized with HN2•Ca, Ca, or were injected with PBS, and were administered with HN2 intraperitoneally following challenge implantation (HN2 administered groups) ; "HN2•Ca-HN2 group" (Fig. 3), "Ca-HN2 group" (Fig. 4) and "PBS-HN2 group" (Fig. 5). Figs. 6, 7 & 8 show the growth curves of the tumors in the groups of mice which were immunized with HN2•Ca, Ca or were injected with PBS, and were not administered with HN2 after challenge (HN2-non-administered group) ; "HN2•Ca group" (Fig. 6), "Ca group" (Fig. 7) and "PBS group" (Fig. 8). One or two of six mice of each group did not take the challenged tumor

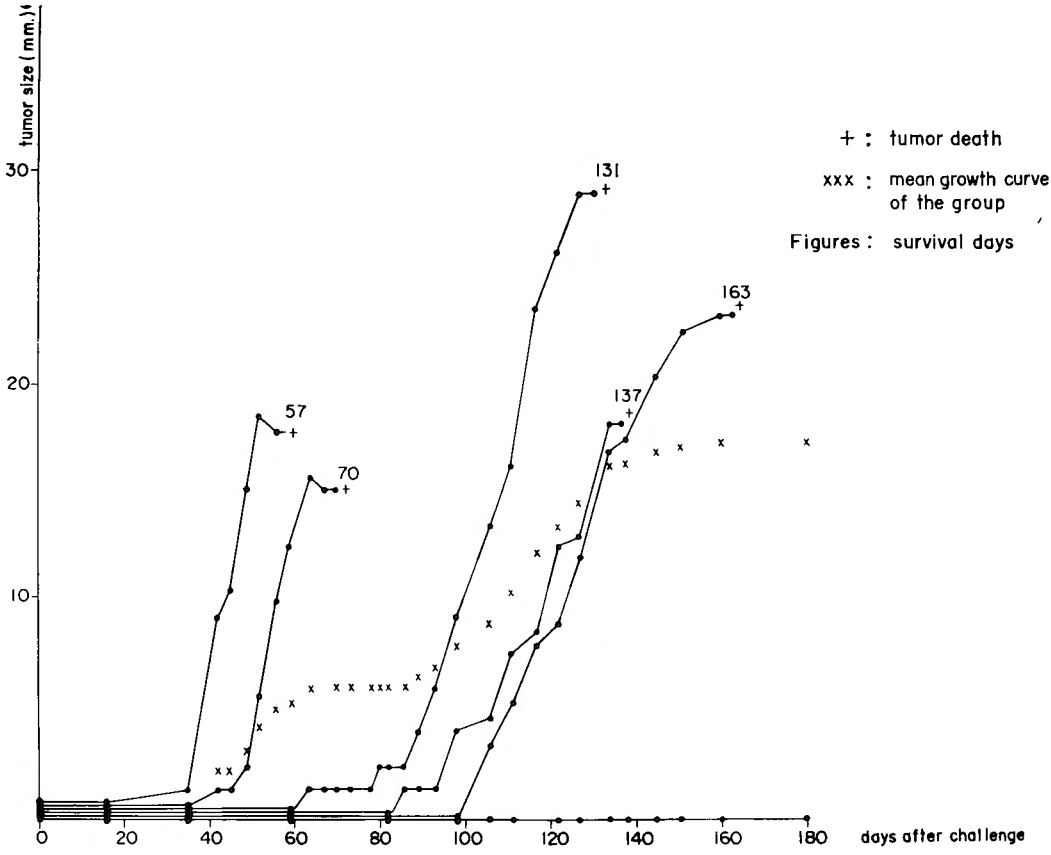

Fig. 3 Growth curve of HN2-administered mice immunized with HN2•Ca (HN2•Ca-HN2 group)

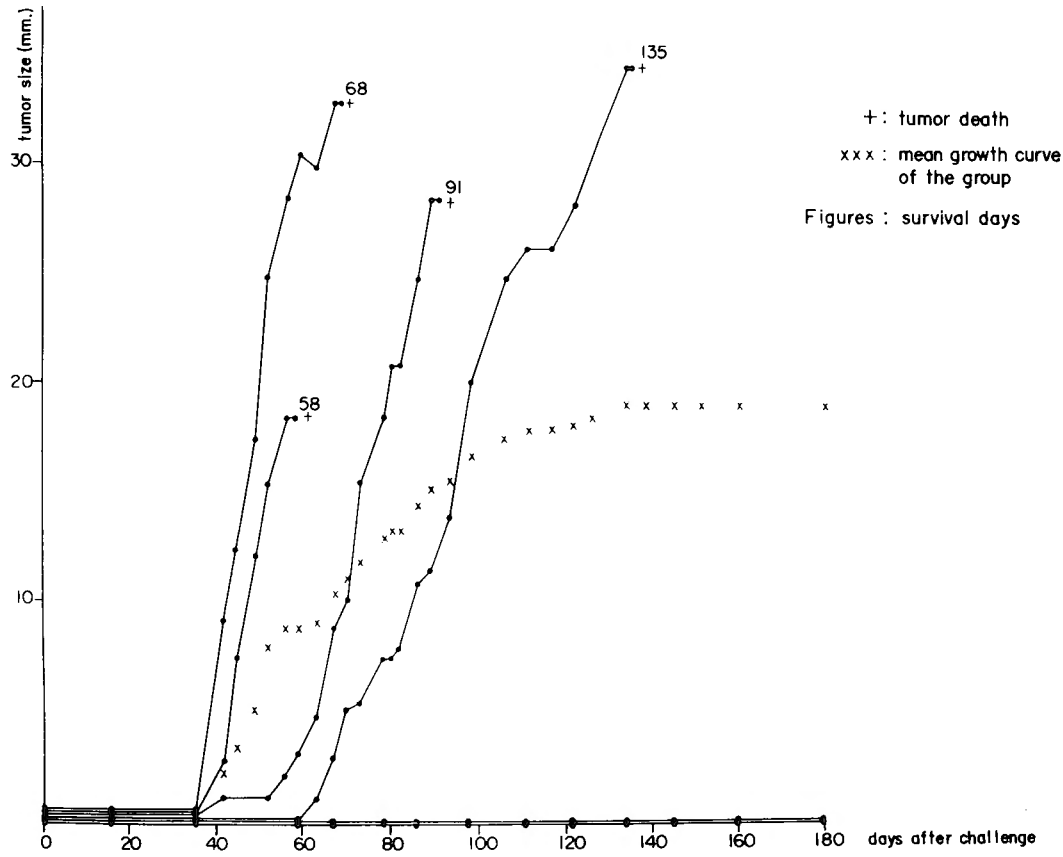

Fig. 4 Growth curve of HN2-administered mice immunized with Ca (Ca-HN2 group)

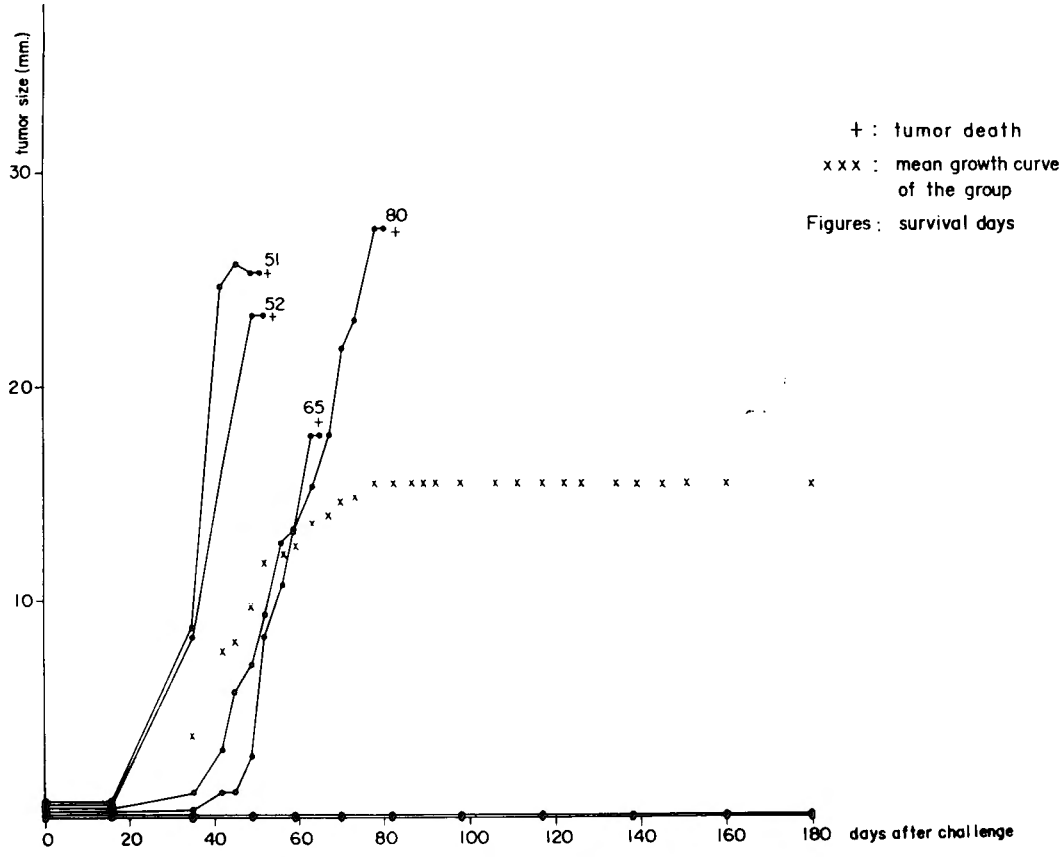

Fig. 5 Growth curve of HN2-administered mice injected PBS instead of cancer tissue (PBS-HN2 group)

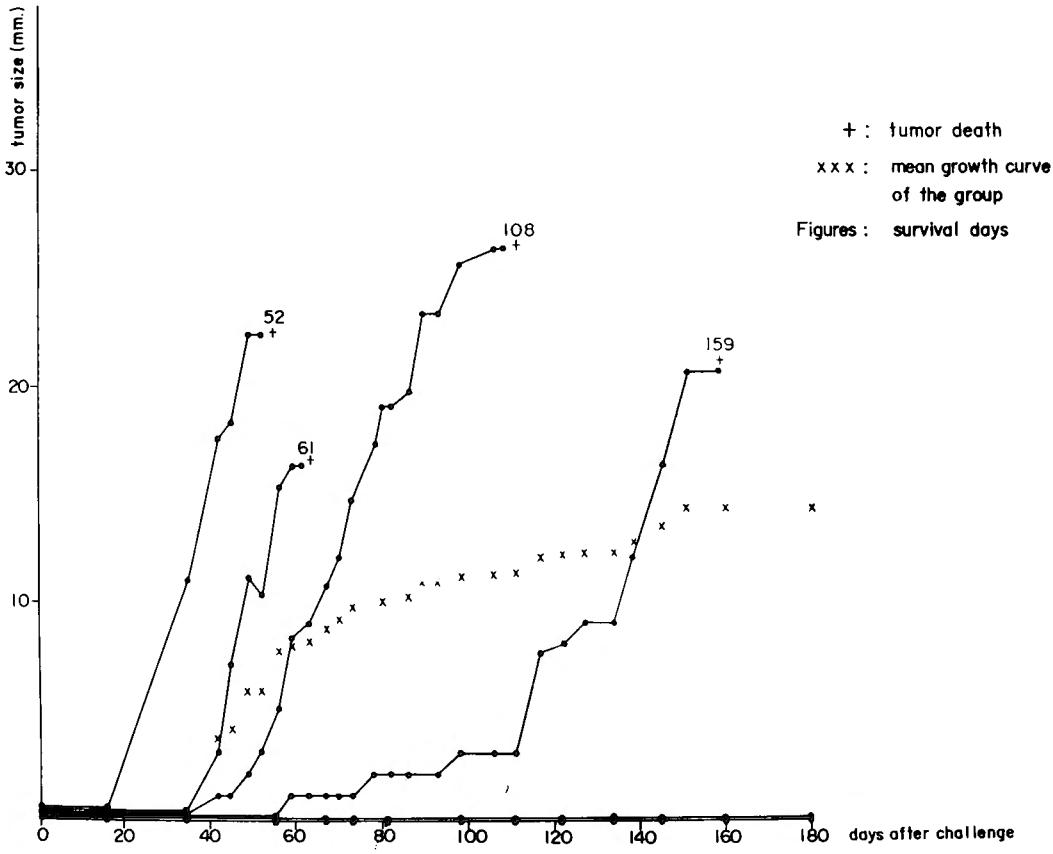

Fig. 6 Growth curve of HN2-non-administered mice immunized with HN2•Ca (HN2•Ca group)

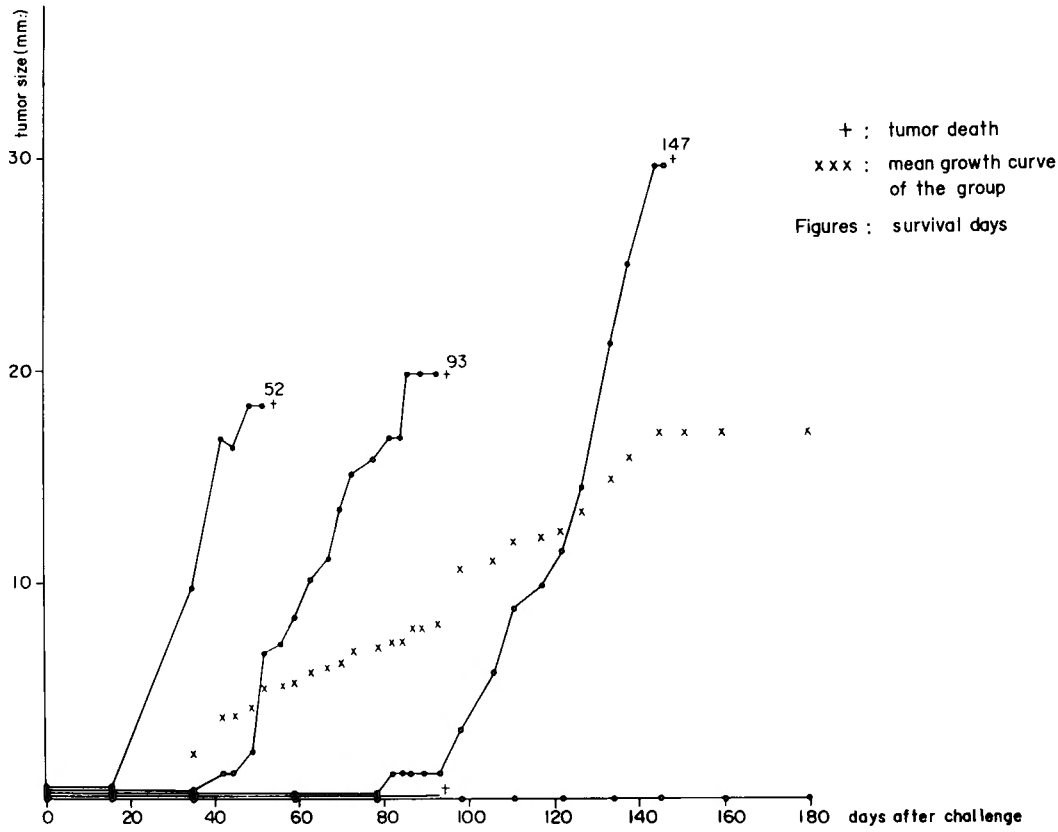

Fig. 7 Growth curve of HN2-non-administered mice immunized with Ca (Ca group)

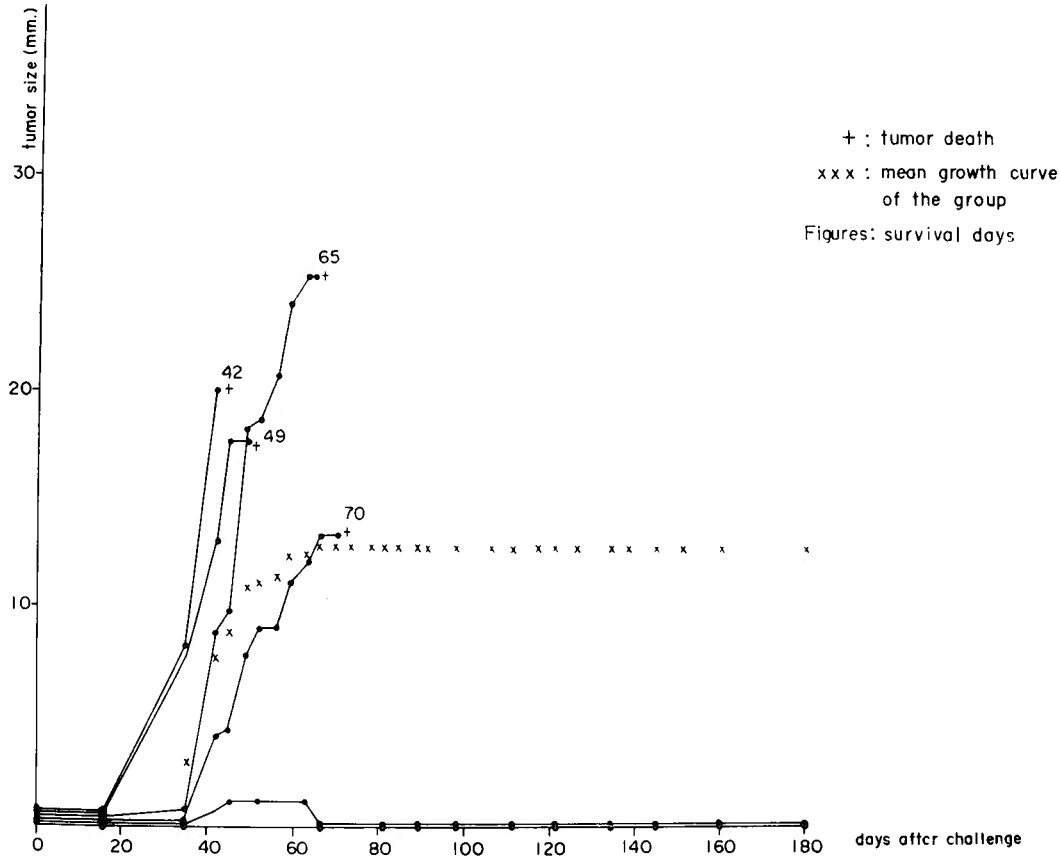

Fig. 8 Growth curve of HN2-non-administered mice injected PBS instead of cancer tissue (PBS group)

cells. Two mice of "Ca group" died accidentally ; one before tumor challenge and another, without palpable tumor, 97 days after the challenge. The onset of palpable tumors was most delayed in "HN2•Ca-HN2 group", that is, 63.2 days after challenge on an average as shown in Table 1. The onsets of tumors in the other groups began at 46.3, 36.8, 43.8, 42.4 and 36.8 days in "Ca-HN2 group", "PBS-HN2 group", "HN2•Ca group", "Ca group" and "PBS group", respectively.

Mean growth curves of each group are shown in Figs. 9 & 10. The curves of "HN2•Ca-HN2 group" and "Ca HN2 group" or "HN2•Ca group" and "Ca group" shift to the right of those of "PBS-HN2 group" or "PBS group", respectively. The mean growth curve

Table 1 Time of tumor onset (days after challenge) in HN2-administered and -non-administered group immunized with HN2•Ca, Ca or PBS

Immunization with	HN2-administered mice		HN2-non-administered mice	
	Time of tumor onset	Mean value	Time of tumor onset	Mean value
HN2•Ca	35, 42, 59, 82, 98	63.2	35, 42, 42, 56	43.8
Ca	42, 42, 42, 59	46.3	35, 42, 80	42.4
PBS	35, 35, 35, 42	36.8	35, 35, 35, 42	36.8

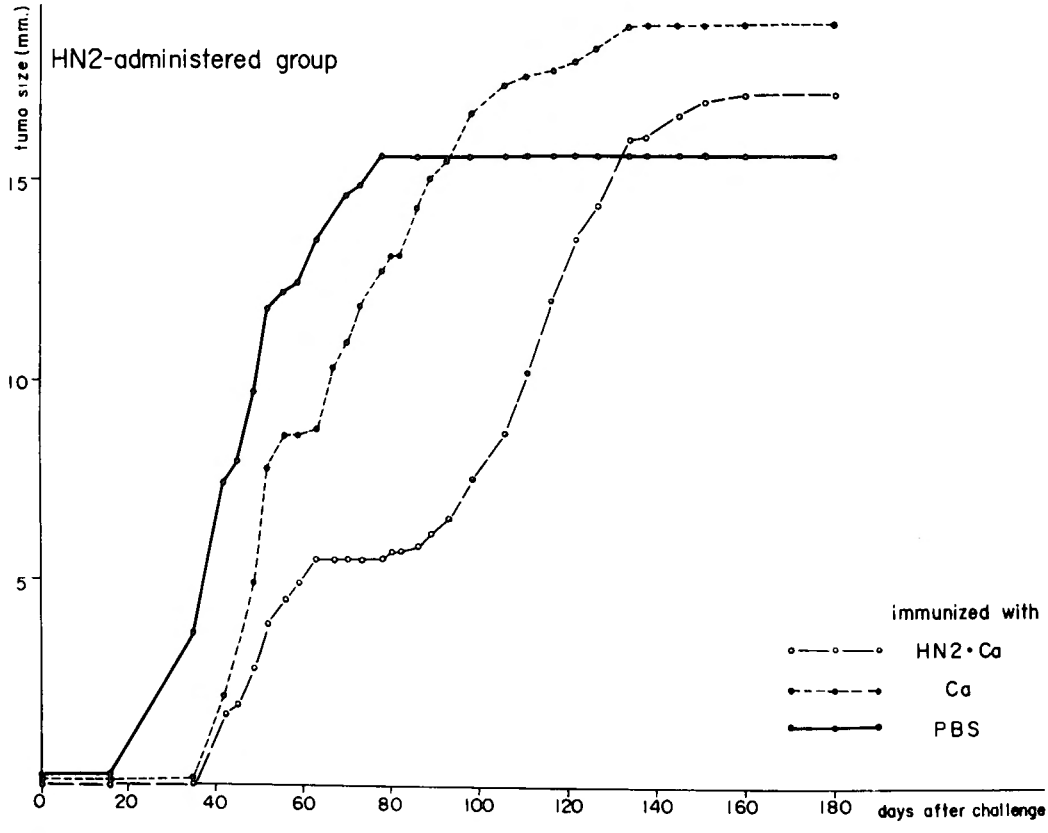

Fig. 9 Mean growth curve of HN2-administered group immunized with HN2•Ca, Ca or PBS

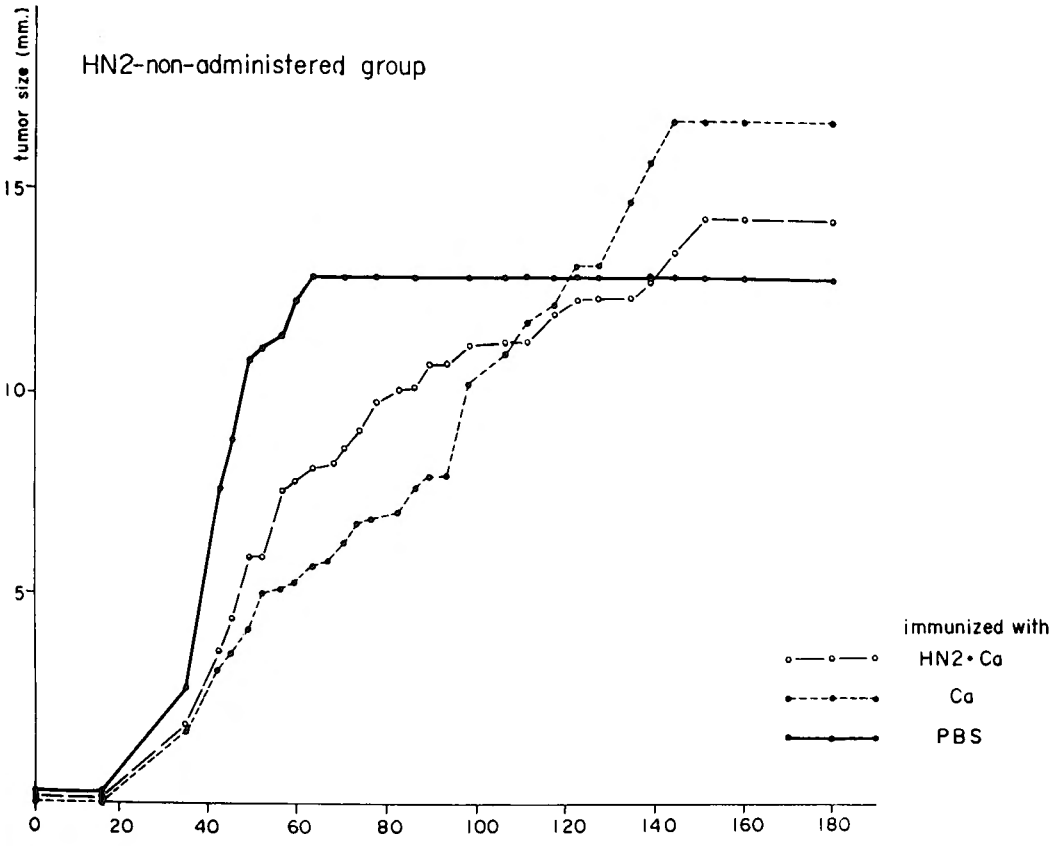

Fig. 10 Mean growth curve of HN2-non-administered group immunized with HN2•Ca, Ca or PBS

of "HN2•Ca group", mice which were immunized with HN2•Ca preparation and were administered with HN2 after tumor challenge, is markedly suppressed compared with those of the other groups. Suppression of growth curves of "HN2•Ca group", "Ca-HN2 group" and "Ca group" are not so strong. The mean value of tumor size of each group on 86 days after challenge is 5.8mm in "HN2•Ca HN2 group", 14.4mm in "Ca-HN2 group", 15.6mm in "PBS-HN2 group", 10.1mm in "HN2•Ca group", 7.8mm in "Ca group" and 12.7mm in "PBS group", respectively (Table 2). The growth of the tumors in "HN2•Ca HN2 group" is markedly suppressed compared with that in "HN2•Ca group", although growth of the tumors in "Ca-HN2 group" or "PBS-HN2 group" of the HN2-administered groups seems to be enhanced when compared with the corresponding groups of the HN2-non-administered groups. Table 3 shows the time at which mean value of tumor size of each group reached 8 mm. "HN2•Ca-HN2 group" shows a significantly high value compared with the other groups. Figs. 11 & 12 show survival days of mice of each group. Survival days of "HN2•Ca HN2 group" is not significantly extended.

Table 2 Tumor size in HN2-administered and -non-administered group immunized with HN2•Ca, Ca or PBS

Immunization with	Tumor size (mm.) at 86 days after challenge					
	HN2-administered mice			HN2-non-administered mice		
HN2•Ca	T. D.	(17.7)	((5.8))	T. D.	(22.3)	((10.1))
	T. D.	(15.0)		T. D.	(16.3)	
	2×2×2	(2.0)		26×21×12	(19.7)	
	(−)			2×2×2	(2.0)	
	(−)			(−)		
	(−)			(−)		
Ca	T. D.	(18.3)	((11.4))	T. D.	(18.3)	((7.8))
	T. D.	(32.7)		25×25×9	(19.7)	
	35×25×11	(21.7)		1×1×1	(1.0)	
	11×10×8	(10.7)		(−)		
	(−)			(−)		
	(−)					
PBS	T. D.	(25.3)	((15.6))	T. D.	(20.0)	((12.7))
	T. D.	(23.3)		T. D.	(17.7)	
	T. D.	(17.7)		T. D.	(25.3)	
	T. D.	(27.3)		T. D.	(13.3)	
	(−)			(−)		
	(−)			(−)		

T. D. : tumor death
( ) : tumor size
(( )) : mean value of the tumor size of each group

Table 3 Days after challenge, mean diameter of each group reaching 8 mm

Immunization with	HN2-administered	HN2-non-administered
HN2•Ca	93	54
Ca	50	73
PBS	40	41

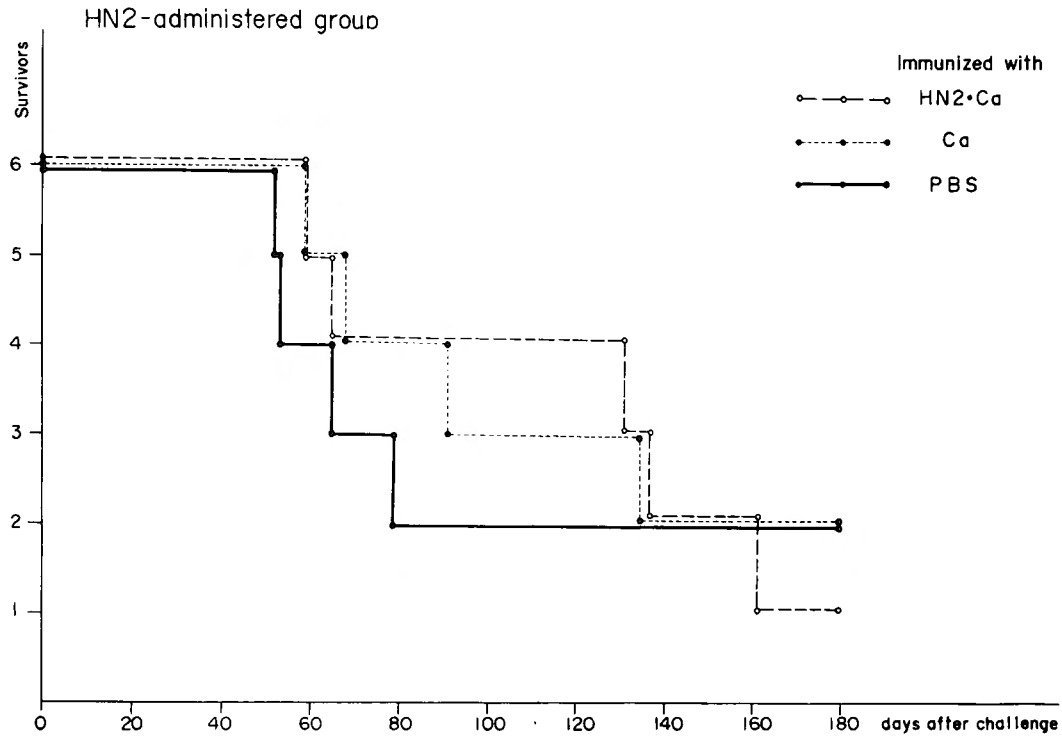

Fig. 11 Survival days of HN2-administered group immunized with NH2•Ca, Ca or PBS

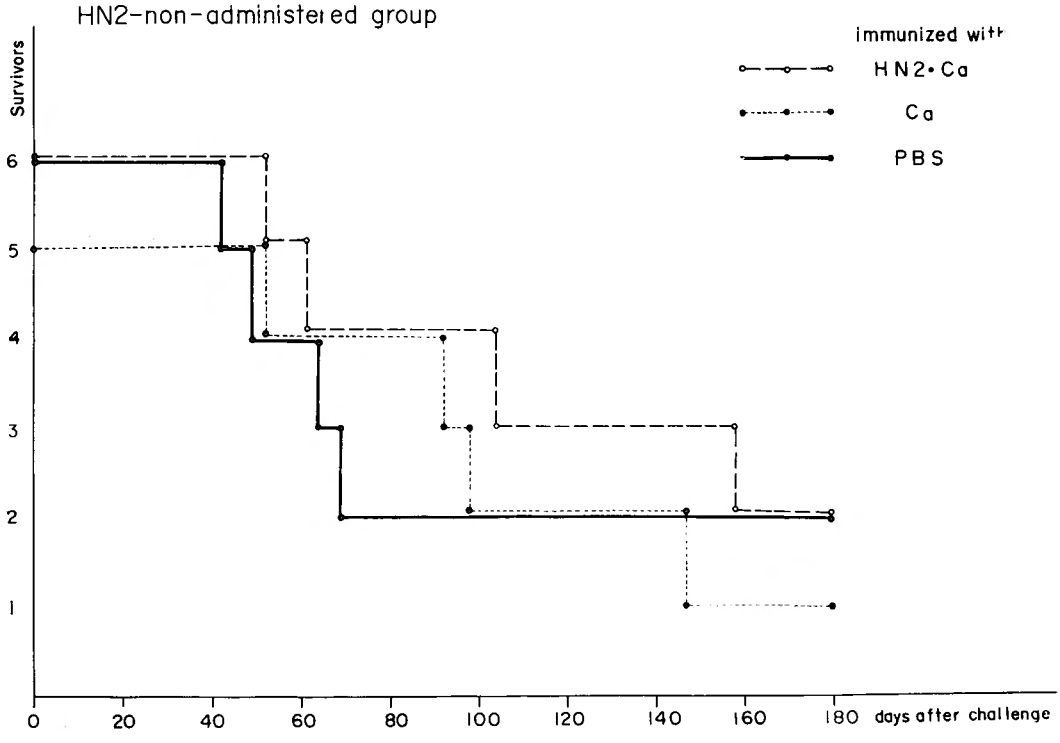

Fig. 12 Survival days of HN2-non-administered group immunized with HN2•Ca, Ca or PBS

DISCUSSION

Does the host react immunologically to his own cancer cells? It has recently been confirmed that chemically induced tumors such as methylcholanthrene-induced sarcoma have immunizing potency to the primary animals in which the tumors originated[8)12)17)18)19)24)28)] and virus tumors such as polyoma virus-[12)24)] or shope papilloma virus-induced tumor[7)] are also antigenic to isologous hosts. However, tumors that spontaneously originated in animals have little immunizing potency to the primary hosts[8)19)24)] or animals of the same inbred strain[11)13)17)]. It seems likely that active autoimmunization with a spontaneous tumor may hardly induce any rejection of the tumor or inhibition of its growth and that the host may be difficult to recognize the tumor as "not self" unless some antigenic changes occur in the tumor tissue.

Attempts to modify or convert the antigenic structure of cancer cells in order to give them antigenicity to the host have been reported[10)14)15)20)26)]. RIGGS[20)] implanted fragments of mammary carcinoma of a dog into mice and returned the implants to the dog, which was followed by complete disappearance of the original lesions. STÜCK[26)] attempted antigenic conversion of established leukemia cells by using an unrelated leukemogenic virus. ONO[15)] induced active immunity in mice against isologous mammary tumor by immunizing the animals with an antigen which was made by means of in vivo conjugation of heterologous serum protein with autolysing tissue of the tumor. NAGAMATSU[14)] reported that active immunization of mitomycin-prepared Ehrlich ascites carcinoma was specifically effective to control growth of the carcinoma when mitomycin was administered after the immunization and tumor challenge. There is also an idea[27)] in the field of autoimmune diseases that some morbid processes may affect antigenic structure of some tissue, resulting in stimulating antibody production against the altered antigen. Some clinical cases of spontaneous regression of cancer[6)28)] may probably be due to the similar mechanism. It is reported[4)5)] that quinidine induced-thrombocytopenic purpura develops through "hapten" mechanism. It seems quite likely, as Dr. DAMESHEK described[4)], that a) combination of quinidine and platelet causes modification of the cell which now becomes antigenic, b) successive administration of drug results in increasing antigenicity and increasing development of specific antibody, c) when sufficient antibody is present and the drug is given again, this results in thrombocytopenia.

Previously, it was proved[1)] that nitrogen mustard (HN2) had a capacity to endow a new antigenic property to an antigen to which HN2 is apt to combine. The antibodies against HN2-treated guinea pig serum cross-reacted with HN2 coupled to an unrelated protein such as rabbit serum or egg albumin. Consequently, it was concluded that HN2 can be one of the determinant groups of immunological specificity of this artificially modified antigen. Therefore, a hypothesis was advanced that active immunization of conjugated cancer tissue with HN2 let probably antibody forming cells recognize the altered tissue as "not self" and produce antibodies to the modified antigen. The resultant antibodies may concentrate upon the cancer tissue in the presence of specific hapten i. e. HN2. The present study was undertaken to investigate this working hypothesis and to see whether active immunization of HN2-conjugated cancer tissue can affect growth of the tumor in isologous system when HN2 is administered.

To reduce an influence of immunogenetic difference between tumor and host as far as possible, an inbred strain of animals and newly-derived tumor of the same inbred stock were employed. The data obtained showed that the time of tumor onset was obviously delayed in "HN2•Ca-HN2 group" compared with those of the other five control groups, and mean growth curve was suppressed in "HN2•Ca-HN2 group". Comparing with the results of "Ca-HN2 group", it can hardly be presumed that the immunity induced is influenced by genetic difference between the tumor and the mice. Immunization with HN2•Ca preparation itself did not result in suppression of tumor growth, but it did when HN2 was administered after challenge. Although antibody production against HN2-treated carcinoma was not studied serologically in the present experiment, the results might be explained by "hapten" mechanism and support the possibility of this kind of treatment for cancer. Immunization with tumor cells viable even after being conjugated with hapten and frequent administration of the hapten after challenge may probably result in more effective suppression of tumor growth.

SUMMARY

Based on the fact that nitrogen mustard (HN2) can act as a hapten, the present experiment was undertaken to see whether active immunization with HN2-treated cancer tissue can affect tumor growth in an isologous system when HN2 is administered in combination with the immunization.

A first-transplant-generation tumor of mammary adenocarcinoma which arose spontaneously in a C3H mouse was treated in vitro with HN2. Groups of mice of the same inbred strain were immunized with the HN2-treated or -untreated cancer tissue and they were challenged, 49 days after the first immunization, with tumor cells of the same tumor of the same transplant generation. Immediately after the challenge 0.1 mg per Kg body weight of HN2 was administered to the mice twice at an interval of one day. Significant retardation of the time of tumor onset was noticed in the group of mice immunized with HN2-treated cancer tissue and administered with HN2 after tumor challenge. Immunization with HN2-treated cancer tissue itself did not result in suppression of tumor growth. In the group of mice immunized with untreated cancer tissue, even when HN2 was administered after challenge, no significant difference was found compared with a control group which was not immunized with cancer tissue. It is suggested that the results might be explained by "hapten" mechanism of nitrogen mustard, although antibody production against HN2-treated cancer tissue was not examined serologically.

Acknowledgement: The authors wish to express their sincere appreciation to Professor Dr. ICHIO HONJO who critically reviewed the manuscript.

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和文抄録

ナイトロジエン・マスタード処理癌組織による癌の免疫学的療法の研究

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免疫学的な手段によつて，癌を制圧しようとする試みは，古くから種々研究されているが，未だ，臨床的に応用される程の効果は，期待し難い．これまで実験的にMethylcholanthrene肉腫の如く，化学物質によつて誘発された癌及びポリオーマ，ショープ乳頭腫等のウィルス性腫瘍は，その同系の動物或いはその原発せる宿主に対して，抗原性を有する事が知られているが，一般に，自然発生せる癌に対しては，その宿主が，免疫学的に反応している確証はまだ不充分のようである．例え，この免疫が存在するにしても一般に極めて弱いものと考えられ，癌の免疫療法に応用し得るかどうか疑問である．これに対して，癌細胞の抗原構造を修飾或いは転換して，宿主に抗原性を認知せしめようとする試みが報告されている．Riggsは，犬の乳癌組織片を，一定期間，マウス皮下に移植して後，その移植片を犬の皮下に接種すると，原発巣が完全に消失したと報告し，癌組織片が遺伝的に異なる宿主の中で成長する事により，抗原構造に変化を来したためと説明している．小野は，自家融解した腫瘍組織と異種血清とを，in vivoで混合さす事により，自動免疫を成立せしめた．又，永松は，Homologousな実験系で，マイトマイシン処理エールリッヒ癌の自動免疫は，癌接種後に，マイトマイシンを投与した時に，特異的に効果のある事を報告している．

我々は，先に，アルキル化制癌剤の一つであるナイトロジエン・マスタード（HN2）は，蛋白等と結合することによつて，新しい抗原性を賦与すること，即ちハプテンとなり得ることを報告した．今回の実験は，上記の事実に基いて，HN2で処理した癌組織で免疫すれば，宿主はこの処理抗原を認め，生じた抗体は，担癌個体に投与されたHN2が，癌組織に作用した時に，そこに集中し，癌の発育に影響を及ぼし得るかも知れないという作業仮説に基いて行なつた．C3Hマウスに自然発生した乳癌を，同系のマウスに移植し，移植第一代の腫瘍で実験を行ない，可及的に宿主と腫瘍との間の免疫遺伝学的差異の影響を除外する様に計画した．この移植第一代の乳癌（Ca VIII-1）より10%癌細胞浮遊液を作製し，HN2を2×10^{-4}Mの濃度に加え，24時間，37°C，pH7.5に保つて後，ドライアイス中に凍結融解を2回行なつて，HN2処理癌抗原を用意した．かくして得た抗原をFreund's complete Adjuvantと共にC3Hマウスの腹部皮下に免疫し，初回免疫より19日後に，4.2×10^4個のCa VIII-1を背部皮下に接種した．その後隔日に，0.1mg/kgのHN2を2回腹腔内に投与し（HN2-処理癌免疫-HN2投与群），接種癌の出現時期，発育，マウスの生存日数を，HN2処理癌免疫-HN2非投与群，無処理癌免疫-HN2投与或いは非投与群，Adjuvant単独-HN2投与或いは非投与群等と比較したところ，HN2処理癌免疫-HN2投与群に，癌出現時期の著明な延長が認められた．無処理癌免疫-HN2投与群及びHN2処理癌免疫-HN2非投与群には癌出現時期の延長は認められなかつた．尚，意義ある延命効果は認められず，一旦発育した癌の増殖は抑制されなかつた．この結果は，前述の作業仮説を支持するものであり，HN2が免疫化学的な過程を経て，腫瘍の発育に，影響を及ぼしたものと考えられる．この点は，更に今後，HN2処理癌に対する抗体を追求する事によつて明らかにされよう．免疫効果をたかめるために凍結融解を行なわないHN2処理細胞により免疫し，癌接種後にHN2の投与回数を増す事により，更にすぐれた効果が期待出来るのではないかと考えられる．